Remarks/Argument

Per the petition and fee submitted herewith, Applicants hereby extend the period for responding by three months, from February 22, 2005 to May 22, 2005. No further fee is believed due for the filing of this paper. The Commissioner is hereby authorized to charge any further fees which may be due, or credit any overpayments, to Deposit Account No. 50-2719.

Claims 11 and 27 are pending in the application. By operation of this amendment, claims 11 and 27 have been amended. Support for amended claim 27 is found in Fig. 1B.

Based on the above changes and the following remarks, the Applicants respectfully request reconsideration of the claims.

Objection to Specification and Drawings

The Applicants have amended the Specification to include a brief description of Figure 1D. Figure 1 has been amended to correct the second "Fig. 1C" to "Fig. 1D." No new matter has been added by these amendments.

Double Patenting

The Applicants note with appreciation that the Examiner is holding the provisional double-patenting rejection of Claim 11 under US Applications 09/436.265; 09/939,483; 09/939,484; and 09/892,360 in abeyance.

Response to rejection under section 101

Claim 27 remains rejected under 35 U.S.C. § 101 because the claimed functionally equivalent derivatives of SEQ ID NO. 2 allegedly lack credible, substantial, specific, or well-established utility. The current Office Action states on pg. 2 that the claimed functionally equivalent derivatives of SEQ ID NO. 2 "would not have utility since it would not be a wild-type protein," and "it is not understood how altering non-critical residues can provide any information as to structure-function relationships." The Applicants respectfully traverse this rejection.

The TWIK-1 protein of SEQ ID NO: 2, as recited in claim 11, has been found to have utility. See pg. 2 of the Office Action mailed August 5, 2004, which states that a specific, substantial and credible utility for the claimed TWIK-1 protein is the screening of potential anti-

arrhythmia agents. The August 5, 2004 Office Action states that this utility is not applicable to the functionally equivalent derivatives of SEQ ID NO. 2 because such derivatives are not naturally occurring.

However, the derivatives of SEQ ID NO. 2 of claim 27 are *functionally equivalent* to the TWIK-1 protein of claim 11. Thus, the claimed functionally equivalent derivatives of claim 27 can also be used to screen potential anti-arrhythmia agents, regardless of whether the claimed derivatives occur naturally in heart tissue. One skilled in the art would expect that anti-arrhythmia agents which are effective on functionally equivalent TWIK-1 derivatives would also be effective on wild-type TWIK-1 proteins – especially, as the Examiner points out, that the claimed derivatives "can be obtained by varying non-critical amino acid residues" (see pg. 2 of the current Office Action). Thus, the same real-world utility which the Examiner has acknowledged for the TWIK-1 protein of claim 11 applies equally to the functionally equivalent derivatives of claim 27.

The Examiner's attention is drawn to pg. 3 of the specification, 2nd paragraph which states that (emphasis added):

> [t]he discovery of this new family of potassium channels ***and cloning*** of a member of this family provides, notably, means for screening drugs capable of modulating the activity of these new potassium channels and thus of preventing or treating the diseases in which these channels are involved.

The specification, beginning on pg. 9, describes the manner in which a TWIK-1 protein coding sequence can be cloned into an expression vector and inserted into Xenopus oocytes for testing of anti-arrhythmia agents. Page 14 of the specification describes a method by which a functionally equivalent TWIK-1 derivative can be cloned and expressed in a cellular host. The paragraph bridging pgs. 14-15 and in the 1st full paragraph of pg. 15 further state that:

> The cells expressing . . . channels exhibiting the properties and structure of the type of the TWIK-1 channels obtained in accordance with the preceding procedures are useful for the screening of substances capable of modulating the activity of the TWIK-1 potassium channels. This screening is carried out by bringing into contact variable amounts of a substance to be tested with cells expressing the . . . potassium channels exhibiting the properties and structure of

> the type of the TWIK-1 channels, then measuring, by any suitable means, the possible effects of said substance on the currents of the potassium channels.
>
> This screening procedure makes it possible to identify drugs useful in the treatment of diseases of the heart . . . for example . . . heart (arrhythmias).

One skilled in the art would understand that the coding sequences of the claimed functionally equivalent TWIK-1 derivatives can be cloned and expressed in Xenopus oocytes in the same manner, to screen for potential anti-arrhythmia agents. The claimed functionally equivalent TWIK-1 derivatives of claim 27 therefore have a specific, substantial, credible and real-world utility, and the rejection of this claim under 35 U.S.C. § 101 should be withdrawn.

Response to rejections under 35 U.S.C. § 112, first paragraph - enablement

Claim 27 has been rejected under 35 U.S.C. § 112, first paragraph for failing to enable functionally equivalent derivatives of the TWIK-1 protein of SEQ ID NO: 2 because the claimed derivatives allegedly lack utility. As demonstrated above, the claimed functionally equivalent TWIK-1 derivatives of claim 27 have the same specific, substantial, credible and real-world utility already acknowledged for the TWIK-1 protein of claim 11, and the rejection of this claim under 35 U.S.C. § 112 should be withdrawn.

Claim 27 has been further rejected under 35 U.S.C. § 112, first paragraph because the Examiner states that there is no requirement that the regions between M1 and P1 and between M2 and M3 are present in the claimed derivatives, and that Applicants have allegedly demonstrated that only the full-length TWIK-1 protein of SEQ ID NO: 2 is functional.

As noted by the Examiner, the functionally equivalent derivatives of SEQ ID NO: 2 as recited in claim 27 "can be obtained by varying non-critical amino acid residues" - see pg. 2 of the current Office Action and pg. 13, 2nd full paragraph of the specification. Although the specification states that the claimed derivatives can be made by "modifying or suppressing one or more amino acid residues," this can be done only "as long as this modification and/or suppression does not modify the functional properties of the TWIK-1 potassium channel of the resultant protein" (see pg. 13, 2nd full paragraph). The 1st full paragraph on pg. 13 states (emphasis added) that

> the object of the present invention is an isolated, purified nucleic acid molecule that codes for a protein constituting a TWIK-1 potassium channel ***or exhibiting the properties and structure of the type of the TWIK-1 channel*** . . .

Thus, one skilled in the art would understand that the claimed functionally equivalent derivatives of TWIK-1 must maintain sufficient structure to allow proper placement and orientation of the conserved P1, P2 and M1-M4 domains in the cell membrane, which is required for TWIK-1 activity. Putative functionally equivalent TWIK-1 derivatives can be obtained by modulating or suppressing non-critical amino acids using routine molecular biology techniques, for example as described on pg. 18 of the speciation. Such derivatives can be readily tested for TWIK-1 activity by expressing the nucleic acids coding for such derivatives into Xenopus oocytes and measuring the K^+ channel activity, as described for example on pgs. 9 and 18-19 of the specification. Once a derivative that is functionally equivalent to TWIK-1 has been identified, this derivative can be used at least to screen potential anti-arrhythmia agents – see the discussion above in the Response to rejection under section 101.

Thus, one skilled in the art would be able to make and use the functionally equivalent TWIK-1 derivatives of claim 27 without undue experimentation, and the Applicants respectfully request withdrawal of the enablement rejection of this claim.

Response to section § 112, second paragraph rejections

Claim 11 is rejected under 35 U.S.C. § 112, second paragraph as being unclear for reciting "consisting essentially of." This phrase has been replaced with "comprising," as suggested by the Examiner.

Claim 27 is rejected under 35 U.S.C. § 112, second paragraph as being unclear for allegedly not reciting the order in which the P1, P2 and M1-M4 regions occur. This claim has therefore been amended to indicated that these regions are arranged in the N- to C-terminal direction as "M1-P1-M2-M3-P2-M4."

The Examiner also suggests that claim 27 be amended to refer to a specific figure which contains the amino acid sequences of the conserved P1, P2 and M1-M4 domains. However, claim 27 already recites SEQ ID NO: 2 as a reference sequence. Page 3, last paragraph of the specification specifies that the amino acid sequence encoded by SEQ ID NO: 2 is set forth in Fig.

1B, and that the P1, P2 and M1-M4 domains are indicated in that figure. MPEP 2173.05(s) states that "[i]ncorporation by reference to a specific figure . . . 'is permitted only in exceptional circumstances where there is no practical way to define the invention in words.'" One skilled in the art would readily understand the amino acid sequences of the conserved P and M domains from reading the specification and reviewing Fig. 1B. Further amendment to claim 27 is therefore not necessary, as the conserved P and M domains are practically defined in the specification and figures.

Claim 27 is further rejected as being unclear because the extent of the amino acid loop between the P1 and M1 domains is allegedly unclear from the figures. The P-domain is a common structural element of K^+ channel proteins, and is considered an essential element of a K^+-permeable pore. See, *e.g.*, the specification at pg. 2, last paragraph. P-domains are well-known in the art, and can be readily identified by those of ordinary skill. Moreover, the conserved M1-M4 domains and the conserved P1 and P2 domain of the claimed functionally equivalent TWIK-1 derivative are respectively circled and underlined in Fig. 1B. Thus, the extent of the M1-M4 and the P1 and P2 domains versus the intervening amino acids is clear from Fig. 1B.

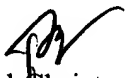
Claim 27 is further rejected because it is allegedly not clear if the phosphorylation consensus site between the M2 and M3 domains encompasses the entire region shown in Fig. 1D. Fig. 1B and pg. 8, 3rd full paragraph identifies the phosphorylation site as a Thr161. One skilled in the art was aware, as of the filing date of the application, that such a phosphorylation consensus site consists of three amino acids, in which the first amino acid is either Ser or Thr, the next amino acid is undefined, and the last amino acid is either Lys or Arg (*i.e.*, S/T-X-K/R; see Fig. 1 of Coqueret et al., 1996, *J. Biol. Chem.* 271(40): 24862-25868, copy enclosed). Thus, one skilled in the art would understand that the phosphorylation consensus site between the M2 and M3 domains is the amino acid sequence "T-R-R," and not the whole sequence between these two domains.

Claims 11 and 27 are therefore clear and definite, and the 35 U.S.C. § 112, second paragraph rejections of these claims should be withdrawn.

Conclusion

The Application is now believed to be in condition for allowance, which action is respectfully requested.

Respectfully submitted,



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DNA Binding by Cut Homeodomain Proteins Is Down-modulated by Protein Kinase C*

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The *Drosophila* and mammalian Cut homeodomain proteins contain, in addition to the homeodomain, three other DNA binding regions called Cut repeats. Cut-related proteins thus belong to a distinct class of homeodomain proteins with multiple DNA binding domains. Using nuclear extracts from mammalian cells, Cut-specific DNA binding was increased following phosphatase treatment, suggesting that endogenous Cut proteins are phosphorylated *in vivo*. Sequence analysis of Cut repeats revealed the presence of sequences that match the consensus phosphorylation site for protein kinase C (PKC). Therefore, we investigated whether PKC can modulate the activity of mammalian Cut proteins. *In vitro*, a purified preparation of PKC efficiently phosphorylated Cut repeats, which inhibited DNA binding. *In vivo*, a brief treatment of cells with calphostin C, a specific inhibitor of PKC, led to an increase in Cut-specific DNA binding, whereas phorbol 12-myristate 13-acetate, a specific activator of PKC, caused a decrease in DNA binding. The PKC phosphorylation sites within the murine Cut (mCut) protein were identified by *in vitro* mutagenesis as residues Thr^{415}, Thr^{804}, and Ser^{987} within Cut repeats 1–3, respectively. Cut homeodomain proteins were previously shown to function as transcriptional repressors. Activation of PKC by phorbol 12-myristate 13-acetate reduced transcriptional repression by mCut, whereas a mutant mCut protein containing alanine substitutions at these sites was not affected. Altogether, our results indicate that the transcriptional activity of Cut proteins is modulated by PKC.

The cDNAs encoding mammalian proteins with sequence homology to the *Drosophila* Cut homeodomain protein have recently been isolated from human, dog, mouse, and rat and were termed, respectively, human CCAAT displacement protein (CDP) or human Cut (hCut),[1] Cut-like homeobox, Cut homeobox, and CCAAT displacement protein 2 (1–5). The terms hCut and murine Cut (hCut and mCut) will be used hereafter in this manuscript. Sequence homology between *Drosophila* and mammalian Cut proteins is limited to five evolutionarily conserved domains: a region predicted to form a coiled coil structure, three repeated regions called Cut repeats (CRs), and a Cut-type homeodomain (HD) (1, 3, 6). We and others have demonstrated that Cut repeats can function as specific DNA binding domains, either independently (CR1 and CR2) or in cooperation with the Cut homeodomain (CR3) (7–10). Therefore, Cut proteins belong to a novel class of homeodomain proteins that contain multiple DNA binding domains: CR1, CR2, and the bipartite Cut repeat 3 homeodomain (CR3HD).

Several lines of evidence suggested that Cut is involved in cell type specification in *Drosophila*. Lethal *cut* mutations resulted in the transformation of external sensory organs into internal (chordotonal) sensory organs (11–13). In contrast, forced expression of *Cut* in embryos transformed internal sensory organs into external sensory organs (14). Moreover, effects caused by *Cut* mutations in other tissues appeared to be the consequence of cells embarking on the wrong developmental program. For example, where the malpighian tubule should normally form, a thickening of the gut wall was observed in *Cut* mutants, suggesting that cells mistakenly differentiated into gut cells (15). Similarly, in the "Cut wing" mutant, cells that should differentiate to form the wing margin instead underwent apoptosis, thereby producing the truncated wing phenotype (12, 16). By analogy with other homeodomain proteins also conserved in evolution, it is believed that Cut proteins play an important role in determining cell type specificity in mammals.

At the molecular level, mammalian Cut homologues have been found generally to act as transcriptional repressors (1, 2, 4, 17). hCut was shown to bind to upstream regulatory sequences of the gp91-*phox* gene, the expression of which coincides with down-regulation of hCut binding activity on differentiation of myeloid cells (17–19). In transient transfection experiments, hCut repressed transcription from the c-*myc* promoter, Cut homeobox-mCut repressed the *Ncam* promoter, and the Cut-like homeobox repressed a reporter construct with a Cut consensus binding site (1, 2, 4, 17).

Several model systems have shown that protein phosphorylation can induce a rapid modulation of transcription activity, providing three main levels of regulation (20). Phosphorylation can affect the DNA binding activity of transcription factors, their subcellular localization, or their interaction with the transcriptional machinery (20–25). Generally, the effects of phosphorylation on DNA binding could be explained either by electrostatic repulsion or by conformational modification of the protein (20). Among the kinases that control these processes, protein kinase C (PKC) plays an important role in the transduction of growth factor signals that lead to either positive or negative change in gene expression, cell growth, and differentiation (26–31). PKC has been shown to be involved in the

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[1] The abbreviations used are: hCut, human Cut; mCut, mouse Cut; CR, Cut repeat; HD, homeodomain; PKC, protein kinase C; GST, glutathione *S*-transferase; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; PMA, phorbol 12-myristate 13-acetate; EMSA, electrophoretic mobility shift analysis; CAT, chloramphenicol acetyltransferase; tk, thymidine kinase.

three main levels of transcription factor regulation described above. For instance, phosphorylation of myogenin by PKC abolished the ability of the protein to bind DNA and consequently inhibited its transcriptional activity (32). By contrast, PKC enhances transcriptional activation by NF-IL6/LAP without having any effect on the DNA binding of the protein (33).

The three evolutionarily conserved regions called Cut repeats were previously shown to function as specific DNA binding domains (7–10). Sequence comparison of Cut repeats from *Drosophila* and mammalian Cut proteins revealed the presence of conserved sequences that match the consensus phosphorylation site for protein kinase C. In the present study, we have investigated whether Cut repeats can be phosphorylated by PKC and whether phosphorylation can affect DNA binding and transcriptional repression by Cut proteins.

MATERIALS AND METHODS

Preparation of Bacterial Fusion Proteins—Plasmid vectors expressing GST Cut fusion proteins were introduced in *Escherichia coli* DH5. Induction of expression and purification of GST proteins were done as described previously (8, 34).

In Vitro Phosphorylation Reactions—In vitro phosphorylation reactions were performed by incubating 50 ng of GST fusion proteins at 37 °C for 30 min in a 20-μl volume containing 2 μl of solution A (200 mM Tris-HCl, pH 7.5, 1 mM $CaCl_2$, 50 mM $MgCl_2$, 0.3% Triton X-100), 1 μl of [γ-^{32}P]ATP (6000 Ci/mmol; Amersham Corp.), and 10 ng of a mixture of purified PKC-α, -β and -γ (Upstate Biotechnology, Inc.). Reactions were terminated by adding 3 μl of loading buffer (2% SDS, 10% glycerol, 50 mM Tris-HCl, pH 6.8, 0.1% bromophenol blue) and boiling for 5 min. Proteins were then resolved on a 10% polyacrylamide gel, followed by autoradiography.

Site-directed Mutagenesis—The GST/CR1 and GST/CR3HD mutants were created by substituting the codon for Thr^{415} and Ser^{987} with codons for Ala by site-directed mutagenesis according to the method of Deng and Nickoloff (35). The nucleotide numbers refer to the sequence of mCut (4) The mCut mutant was created by substituting the codon for Ser^{402}, Thr^{415}, Ser^{789}, Thr^{804}, Ser^{972}, and Ser^{987} with codons for Ala. Mutations were confirmed by DNA sequencing.

Cell Culture—Cells were grown in DMEM supplemented with 10% fetal bovine serum (FBS). HL60 cells were cultured in RPMI 1640 medium supplemented with 20% FBS. When required, the culture medium was changed to DMEM or RPMI 1640 medium containing 0.4% FBS to serum starve the cells. PMA or calphostin C stimulation was performed after 48 h of serum starvation by changing the medium to 0.4% FBS/DMEM or RPMI 1640 medium containing PMA or calphostin C at a concentration of 100 ng/ml or 1 μM, respectively. Cells were further cultured for different times as indicated in the figure legends.

Transient Transfections and Preparation of Cytoplasmic and Nuclear Extracts—NIH 3T3 or COS cells were plated, respectively, at densities of 3×10^5 and 10×10^5 cells/100-mm plates 24 h prior to transfection. All transfection experiments were repeated at least three times. Transient transfection in NIH 3T3 and COS cells were performed using the calcium phosphate precipitation method and the DEAE-dextran procedure, respectively (36, 37). For electrophoretic mobility shift analysis (EMSA), expression vector coding for Cut proteins and PKC-β1 (kindly provided by Dr. Yasutomi Nishizuka) were used at concentrations of 1 and 3 μg, respectively, for COS cells and 6 μg for NIH 3T3 cells. For CAT assays, NIH 3T3 and COS cells were transfected with 5 or 3 μg of the tkCAT reporter construct and 6 or 9 μg of the effector plasmids, respectively. The amount of transfected DNA was kept constant by addition of appropriate amounts of the parental expression vector. Following transfection, cells were serum starved for 2 days in DMEM supplemented with 0.4% FBS. Cells were harvested with 1 ml of TEN buffer (40 mM Tris, pH 7.5, 1 mM EDTA, 150 mM NaCl), and extracts were prepared according to the method of Lee *et al.* (38) with some modifications. Briefly, cell pellets were resuspended in 30 μl of buffer A (10 mM Hepes, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml aprotinin, and 1 mM dithiothreitol). After three cycles of freeze-thaw, cytoplasmic extracts were recovered by centrifugation at 12,000 rpm for 1 min and retrieval of the supernatant. Nuclear pellets were resuspended in 20 μl of buffer C (20 mM Hepes, pH 7.9, 1.5 mM $MgCl_2$, 420 mM KCl, 0.2 mM EDTA, 25% glycerol, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 mM dithiothreitol). Following a 30-min incubation at 4 °C, nuclear extracts were spun down at 12,000 rpm for 5 min, and the supernatants were recovered. Extracts were either used immediately or quick frozen in a dry ice ethanol bath and stored at −80 °C.

EMSAs—Bacterially expressed proteins (50 ng) were preincubated for 5 min at room temperature with 50 ng of poly(dI-dC) in 25 mM NaCl, 10 mM Tris, pH 7.5, 1 mM $MgCl_2$, 5 mM EDTA, pH 8, 5% glycerol, and 1 mM dithiothreitol. Cytoplasmic or nuclear extracts prepared as described above were incubated in the same buffer with either 1.5 μg of poly(dI-dC) for transfected cells or 0.5 μg of a polymerase chain reaction-amplified random oligonucleotide for nontransfected cells (9). In selected experiments, extracts were preincubated for 30 min at room temperature with 2 units of calf intestinal phosphatase, and nonspecific competitors were added for the last 5 min of reaction. A double-stranded nucleotide containing a Cut consensus binding site (upper strand, 5′-AAAAGAAGCTTATCGATACCGT-3′) was end labeled using the Klenow polymerase, and 10 pg of the probe (20,000 cpm) was then added to the protein mixture for 15 min. Samples were then loaded on a 5% polyacrylamide gel (30:1) and separated by electrophoresis at 8 V/cm for 2 h in 50 mM Tris, 0.38 M glycine, and 1 mM EDTA, pH 8.5. Gels were then dried and visualized by autoradiography.

Generation of hCut Antibody—To generate polyclonal antibodies against hCut, rabbits were injected with 500 μg of purified bacterial fusion protein containing amino acids 1102–1505 of hCut, in Freund's complete adjuvant. The animals were boosted twice with 250 μg of protein, and serum was collected 10 days after the last boost. The polyclonal antibodies were purified by affinity chromatography. The serum was passed through two GST affinity columns, and the flow-through was then applied to a GST/Cut affinity column to isolate antibodies against hCut. Monoclonal antibodies against hCut were generated by Molecular Immunogenetics (San Andreas, CA), using the same GST/Cut fusion protein and were prepared as ascetic fluids. The monoclonal antibodies used in the present study, α-Cut (W3) and α-Cut (A), were initially characterized using a purified fraction of hCut (2). The α-Cut (A) and (W3) antibodies recognize epitopes present, respectively, in CR3 and the carboxyl-terminal 200 amino acids (amino acids 1302–1505) of hCut. Both α-Cut antibodies can react with other mammalian Cut proteins (9). Although the α-Cut (W3) antibody does not bind to a DNA binding domain, incubation of this antibody with cellular extracts mainly inhibits Cut DNA binding and produces only a weak supershifted band (2, 9). The weak supershift can be seen on long exposure or when using extracts from transfected cells that express a high level of Cut proteins (see Fig. 6, *lane 7*). Inhibition of DNA binding by the α-Cut (A) and (W3) antibodies is specific to hCut, since DNA binding by other factors is not inhibited.

CAT Assays—Transfected cells were harvested with 1 ml of TEN buffer as described above, centrifuged, and resuspended in 100 μl of 0.25 M Tris, pH 7.5. Cells were then subjected to three cycles of freeze-thaw. Following centrifugation, cellular extracts were recovered and used directly in CAT assays or stored frozen at −80 °C. CAT assays were performed as described (39) and visualized by autoradiography.

Western Blot Analysis—Transfected cells were harvested with 1 ml of TEN buffer as described above, centrifuged, and resuspended in 20 μl of Laemmli buffer. Equal amounts of protein were then boiled for 5 min and loaded on an 8% SDS-polyacrylamide gel. The gel was soaked for 10 min in a solution of 0.1 M Tris, 0.192 M glycine, and 20% (v/v) methanol, and proteins were electrotransferred to nylon membranes for 1 h at 4 °C. Blots were then washed five times with phosphate-buffered saline supplemented with 0.1% Tween 20 and incubated for 1 h at room temperature in phosphate-buffered saline-Tween 20 containing 3% bovine serum albumin to prevent nonspecific binding of the antibody. Following washing, blots were then incubated with a polyclonal antibody directed against Cut diluted in phosphate-buffered saline-Tween 20 buffer. After washings, membranes were incubated with a second antibody conjugated to horseradish peroxidase for 40 min at room temperature. Proteins were then visualized using the ECL system (Amersham) according to the instructions of the manufacturer.

RESULTS

Bacterially Expressed Cut Repeats Are Phosphorylated in Vitro by PKC—Previous studies on mammalian homologues to the *Drosophila* Cut homeodomain protein have revealed that the three regions called Cut repeats function as specific DNA binding domains. Sequence comparison of Cut repeats indicated the presence of potential phosphorylation sites for PKC (Fig. 1). These sites are located at the same position within each Cut repeat and are conserved among mammals (human, mouse, rat, and dog) and in *Drosophila* (Fig. 1). To determine

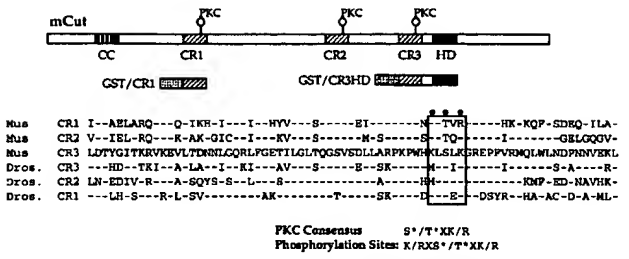
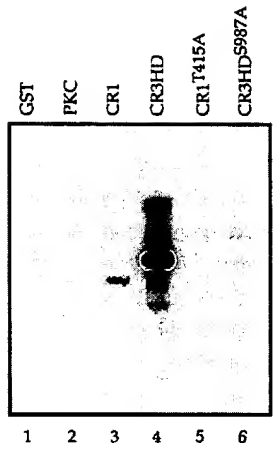


FIG. 1. **Conserved PKC consensus sites in the Cut repeat sequences.** A representation of the mCut protein is displayed at the *top*. *Boxes,* evolutionarily conserved domains: the coiled-coil (*CC*), CRs, and HD. Below are shown the protein segments present in the GST fusion proteins. The sequences of the three Cut repeats from the *Drosophila* (*Dros.*) and mCut (*Mus*) proteins are aligned, the sequences that match the consensus PKC phosphorylation sites are *boxed*, and the PKC specificity determinants are indicated by the *dots* at the *top*. Note that mammalian Cut proteins all share identical amino acid sequences within their Cut repeats.

whether these sites within the mCut protein can be phosphorylated by PKC, GST/CR fusion proteins were incubated *in vitro* in the presence of radiolabeled γ-ATP and a purified preparation of PKC-α, -β, and -γ. GST/CR1 and GST/CR3HD were both phosphorylated, whereas GST alone was not (Fig. 2, compare *lanes 3* and *4* with *1*), indicating that PKC phosphorylation involved the Cut repeat moiety. CR3HD appeared to be a better substrate than CR1, as it produced a stronger signal on phosphorylation, yet the two proteins were present in similar amounts, as judged from Coomassie Blue staining (data not shown). To confirm the identity of PKC phosphorylation sites, *in vitro* mutagenesis was performed, in which Thr^{415} and Ser^{987} within CR1 and CR3 were substituted with alanines in $GST/CR1^{T415A}$ and $GST/CR3HD^{S987A}$, respectively. As shown in Fig. 2, *lanes 5* and *6*, phosphorylation of the mutated fusion proteins was reduced to background levels. Minor phosphorylated species in *lane 4* most likely represent shorter GST/CR3HD fusion proteins, since these species are not observed using the mutated GST/CR3HD.

Phosphorylation of Cut Repeats by PKC in Vitro Inhibits DNA Binding—The addition of a negative charge within Cut repeat DNA binding domains would be predicted to cause a reduction in DNA binding. To verify this, GST/CR fusion proteins were incubated with purified PKC in the presence or absence of cold ATP, and EMSAs were performed using oligonucleotides encoding a consensus Cut binding site. Incubation in the presence of PKC and cold ATP significantly reduced the DNA binding activity of both GST/CR1 (Fig. 3, compare *lanes 1* and *2*) and GST/CR3HD (Fig. 3, compare *lanes 3* and *4*). In contrast, DNA binding by the mutated $GST/CR3HD^{S987A}$ was not inhibited, suggesting that the effect of PKC on CR3HD is mediated through phosphorylation of Ser^{987} (Fig. 3, compare *lanes 5* and *6*). Similar experiments using $GST/CR1^{T415A}$ were inconclusive, since this mutated protein fails to bind DNA (data not shown).

Cut DNA Binding Activity in Cellular Extracts Is Increased by Treatment with Phosphatase—We then asked whether DNA binding by mammalian Cut proteins could be affected *in vivo* by their phosphorylation state. Nuclear extracts prepared from various cell lines were treated with calf intestinal phosphatase and then analyzed by EMSA using a consensus Cut binding site (Fig. 4). Only one retarded band was detected with this binding site. This protein-DNA complex is specific for Cut proteins, since it disappeared when anti-Cut monoclonal antibodies were included in the reaction (Fig. 4, *lanes 3, 6, 9,* and *12*), whereas a nonspecific monoclonal antibody directed against hemagglutinin had no effect (Fig. 4, *lane 13*). An increase in Cut DNA binding was observed with each extract



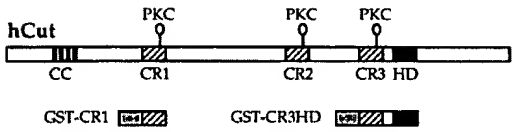


FIG. 2. **PKC efficiently phosphorylates GST/CR1 and GST/CR3HD *in vitro* but not $GST/CR1^{T415A}$ and $GST\text{-}CR3HD^{S987A}$.** GST/Cut repeat fusion proteins (50 ng) were incubated for 30 min at 37 °C in the presence of 20 ng of protein kinase C (α, β, and γ isozymes) and radiolabeled γ-ATP. Samples were then separated by electrophoresis on a SDS-polyacrylamide gel. $GST/CR1^{T415A}$ and $GST\text{-}CR3HD^{S987A}$ correspond, respectively, to mutated GST-CR1 and GST-CR3HD proteins in which Thr^{415} and Ser^{987} have been replaced by alanine. The amino acid numbering refers to that of mCut.

following phosphatase treatment. A similar increase in DNA binding, although of a lower magnitude, was detected using an extract from COS cells that had been transfected with a vector expressing the murine Cut protein (Cut homeobox-mCut). These results suggests that a proportion of Cut proteins exist in a phosphorylated state in cells and that dephosphorylation increases their DNA binding activity.

Treatment of Cells with Calphostin C, an Inhibitor of PKC, Increases DNA Binding by Cut—To verify whether PKC is responsible for the phosphorylation of Cut proteins *in vivo*, cells were incubated for 30 min in the presence of calphostin C, a specific inhibitor of PKC, and nuclear extracts were subsequently tested in EMSA (Fig. 5). Following treatment with calphostin C, Cut-specific DNA binding was increased (Fig. 5, compare *lanes 2, 3, 5,* and *8*), suggesting that PKC is, at least in part, responsible for the phosphorylation of Cut proteins. The increase in DNA binding activity is greater with phosphatase than with calphostin C (Fig. 5, compare *lanes 1* and *3* with *6* and *8*). It is possible that calphostin C does not completely inhibit phosphorylation by PKC. Alternatively, as suggested by the results of other experiments, it is likely that other kinases, which are not inhibited by calphostin C, are involved in the phosphorylation of Cut repeats.[2]

Treatment of Cells with PMA, an Activator of PKC, Decreases DNA Binding by Cut—We investigated whether treatment of cells with PMA, an activator of PKC, had an effect on Cut DNA binding. The stimulation of PKC by PMA is generally performed on cells synchronized in G_0; however, we found that Cut-specific DNA binding is virtually undetectable in quiescent cells (Fig. 4 and data not shown). We therefore used a different approach. COS cells were cotransfected with a vector coding for

[2] O. Coqueret and A. Nepveu, manuscript in preparation.

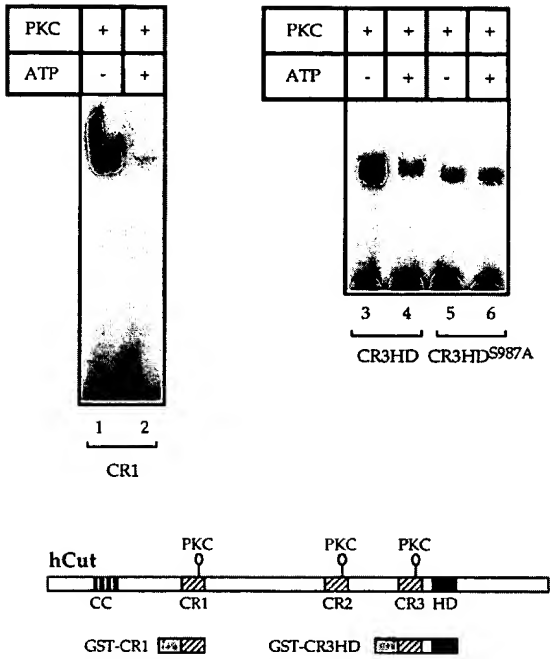


FIG. 3. **Phosphorylation of Cut repeats by PKC reduces DNA binding by wild type GST/Cut repeat fusion proteins but not by GST-CR3HD^{S987A}.** GST/Cut repeat fusion proteins (50 ng) and 20 ng of protein kinase C (α, β, and γ isozymes) were incubated for 30 min at 37 °C in the presence or absence of cold ATP. Samples were then incubated, at room temperature for 15 min, with radiolabeled oligonucleotides encoding a consensus Cut binding site. DNA-protein complexes were resolved on a nondenaturing polyacrylamide gel.

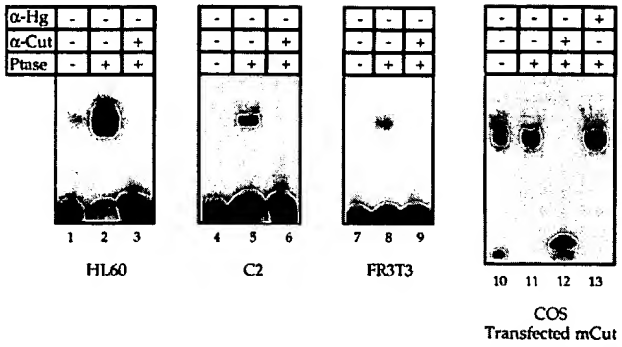


FIG. 4. **Effect of phosphatase on DNA binding by mammalian Cut proteins.** Nuclear extracts were prepared from cells that had been maintained for 4 (HL60, C2, and FR3T3) or 2 (COS) days in DMEM plus 0.4% calf serum. COS cells were transfected with 3 μg of vector DNA expressing the whole mCut protein; the next day the medium was changed for DMEM plus 0.4% calf serum, and 2 days later cells were harvested. Nuclear extracts (5 μg of proteins) were incubated for 30 min at room temperature in the presence or absence of 2 units of calf intestinal phosphatase (*Ptase*) as indicated. The protein samples were then incubated with radiolabeled oligonucleotides encoding a consensus Cut binding site in the presence or absence of monoclonal antibodies directed against either Cut (*α-Cut*) or hemagglutinin (*α-Hg*) (2, 9). DNA-protein complexes were resolved on a nondenaturing polyacrylamide gel.

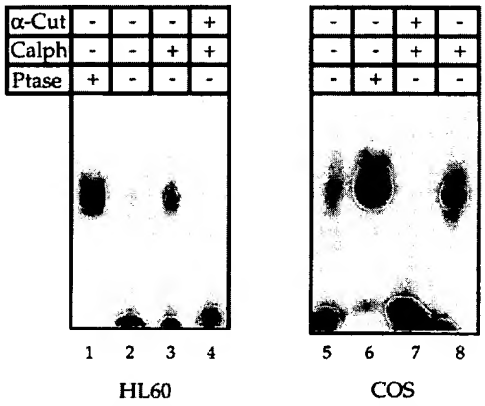


FIG. 5. **Treatment of cells with calphostin, an inhibitor of PKC, increases DNA binding by Cut proteins.** Calphostin (*Calph*) was added to the medium at a concentration of 1 μM (*lanes 3, 4, 7,* and *8*). Following a 30-min incubation, cells were harvested, and nuclear extracts were prepared. For comparison, in *lanes 1* and *6* nuclear extracts (5 μg of proteins) from untreated cells were incubated for 30 min at room temperature in the presence or absence of 2 units of calf intestinal phosphatase (*Ptase*) as indicated. All samples (5 μg) were incubated at room temperature for 15 min with radiolabeled oligonucleotides encoding a consensus Cut binding site, in the presence or absence of a monoclonal anti-Cut antibody (*α-Cut*) as indicated (2, 9). DNA-protein complexes were resolved on a nondenaturing polyacrylamide gel.

the PKC-β1 isoform and a vector expressing either the entire murine Cut protein or only the CR3HD region. The next day, the medium was changed for DMEM containing 0.4% calf serum. Forty eight hours later, PMA was added to the medium and after 30 min, and nuclear extracts were prepared and tested in EMSA. DNA binding by either CR3HD or the whole mCut was reduced following a short treatment of cells with PMA (Fig. 6, compare *lane 1* with *2*, and *lane 5* with *6*). In contrast, even 4 h after addition of PMA to the medium, the steady-state level of the mCut protein was not reduced (see Fig. 8*B*). These results suggest that stimulation of PKC in cells leads to phosphorylation of Cut repeats and down-modulation of DNA binding.

We next tested the effect of PMA treatment on DNA binding by Cut proteins with PKC phosphorylation sites that had been mutated. As shown in Fig. 7, when COS cells were transfected with a vector expressing the mutated CR3HD^{S987A} protein, the treatment of cells with PMA did not cause a decrease but instead led to an increase in DNA binding (Fig. 7, *lanes 5* and *6*). Western blot analysis confirmed that the level of CR3HD^{S987A} protein did not change following PMA treatment (data not shown). The fact that DNA binding of CR3HD^{S987A} was not reduced strongly implies that Ser^{987} is indeed the target of the PKC-mediated modulation. However, the increase in DNA binding was a surprising result. It could be accounted for if we assume that another kinase can phosphorylate and down-modulate CR3HD DNA binding and that PKC stimulation leads to the activation of a phosphatase that removes all phosphate groups, thereby increasing DNA binding. Indeed, dephosphorylation of DNA binding regulatory sites after activation of PKC has been reported in the case of the c-Jun protein (40). This hypothesis is further supported by the fact that Cut repeats contain sequences that match the consensus phosphorylation site for casein kinase II: SVSD. Preliminary experiments indicate that casein kinase II can efficiently phosphorylate Cut repeats *in vitro*, leading to a reduction in DNA binding.[3] To verify this hypothesis, we mutated the casein kinase II and PKC phosphorylation sites within each Cut repeat and tested the effect of PKC stimulation on DNA binding by such a mutated protein, which we refer to as mCut^{6A}. Treatment of transfected cells with PMA did not affect DNA binding by the mCut^{6A} protein (Fig. 7, *lanes 3* and *4*). In summary, our results indicate that stimulation of PKC following treatment of cells with PMA causes a decrease in DNA binding by the wild type mCut protein and CR3HD derivative (Fig. 6). In contrast, no decrease in DNA binding was observed

[3] O. Coqueret and A. Nepveu, manuscript in preparation.

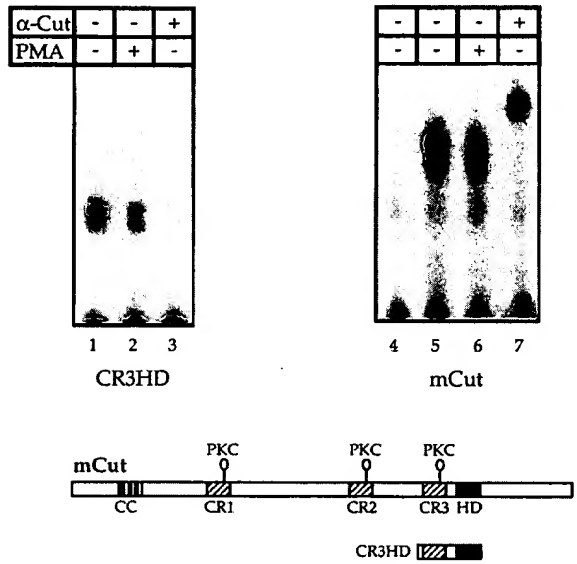


FIG. 6. **Treatment of cells with PMA, an activator of PKC, reduces DNA binding by Cut proteins.** Extracts were prepared from untransfected (*lane 4*) or transfected (all other *lanes*) COS cells. COS cells were transfected with 1 μg of vector DNA expressing either the CR3-HD protein or the full-length murine Cut protein (mCut), together with 3 μg of a vector expressing the β1 subunit of PKC. The next day, the medium was changed for DMEM containing 0.4% calf serum. Forty eight hours later, PMA (100 ng/ml) was added to the medium, and 30 min later nuclear extracts were prepared. Extracts (5 μg) were incubated at room temperature for 15 min with radiolabeled oligonucleotides encoding a consensus Cut binding site. When indicated, incubation was done in the presence of monoclonal anti-Cut antibodies, α-Cut (A) and α-Cut (W3) in *lanes 3* and *7* respectively (2, 9). The α-Cut (A) and (W3) antibodies recognize epitopes present, respectively, in CR3 and the carboxyl-terminal region of mammalian Cut proteins. DNA-protein complexes were resolved on a nondenaturing polyacrylamide gel. Note that the human and murine Cut proteins share identical Cut repeat sequences.

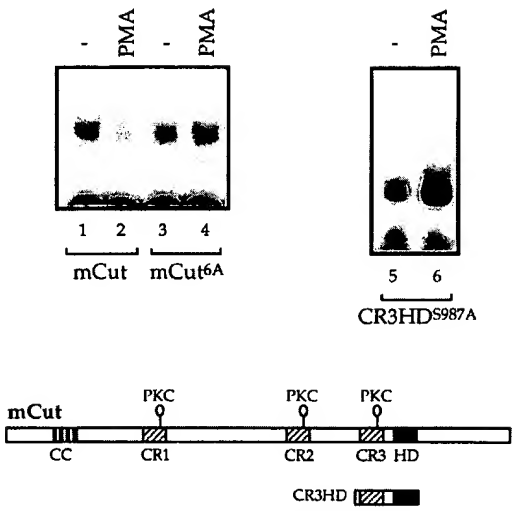


FIG. 7. **Treatment of cells with PMA, an activator of PKC, does not modify DNA binding by mutated Cut proteins.** NIH 3T3 cells were transfected with 5 μg of vector DNA expressing either the wild type mCut, CR3HD, or the mutated forms of these proteins ($mCut^{6A}$ and $CR3HD^{S987A}$), together with 6 μg of a vector expressing the β1 subunit of PKC. Forty eight hours later, PMA (100 ng/ml) was added to the medium, and following a 30-min incubation, cells were harvested, and nuclear extracts were prepared. Extracts (5 μg) were incubated at room temperature for 15 min with radiolabeled oligonucleotides encoding a consensus Cut binding site, in the presence or absence of a monoclonal anti-Cut antibody (α-Cut) as indicated (2, 9). DNA-protein complexes were resolved on a nondenaturing polyacrylamide gel.

using a $CR3HD^{S987A}$ protein or a mutant mCut protein containing alanine substitutions at the six putative PKC and casein kinase II phosphorylation sites within Cut repeats (Fig. 7). These results suggest that stimulation of PKC *in vivo* leads to phosphorylation of Cut repeats and down-modulation of DNA binding.

PKC Activation Inhibits Cut Repression Activity in Vivo—Results from several groups have revealed that mammalian Cut proteins function as transcriptional repressors (2, 4, 17, 19). We therefore decided to verify whether stimulation of PKC could affect repression by Cut proteins. The tkCAT reporter plasmid represents a good model for repression by Cut, since expression from the minimal herpes simplex virus-1 tk promoter was shown to depend on the presence of CCAAT and Sp1 binding sites, both of which are recognized by Cut proteins (18, 26, 32, 45, 50). COS and NIH 3T3 cells were cotransfected with the tkCAT reporter plasmid together with vectors expressing the PKC-β1 isoform as well as wild type or mutated Cut proteins. Two days later, cells were treated for 4 h with PMA, and expression of the reporter plasmid was measured by CAT assays. PMA alone had no effect on expression (Fig. 8, *lanes 1* and *3*). Inclusion of the Cut-expressing vector in the transfection mix, as expected, led to a reduction in expression (Fig. 8, *lanes 2* and *7*). Repression by Cut either was reduced (NIH 3T3 cells; Fig. 8, *lanes 7* and *8*) or completely abolished (COS cells; Fig. 8, *lanes 2* and *4*) following treatment with PMA. Western blot analysis demonstrated that the steady-state level of the mCut protein did not decrease during this period (Fig. 8*B*), suggesting the involvement of posttranslational mechanisms in the modulation of Cut activity.

We then asked whether PKC stimulation would similarly affect repression mediated by the $mCut^{6A}$ protein. As shown in Fig. 8, *lane 9*, $mCut^{6A}$ was capable of repressing the tkCAT reporter plasmid, although to a lesser extent than the wild type protein. However, PKC activation did not prevent transcriptional repression by the $mCut^{6A}$ protein (Fig. 8, *lane 10*). We conclude that down-modulation of Cut transcriptional repression activity after PKC activation requires the presence of the conserved PKC phosphorylation sites within Cut repeats. Altogether, these results indicate that stimulation of PKC leads to a reduction in transcriptional repression by Cut proteins.

DISCUSSION

In this study, we have investigated the functional significance of putative PKC phosphorylation sites present within the recently identified Cut repeat DNA binding domains. We have shown that Cut repeats are phosphorylated *in vitro* by purified preparations of PKC-α, -β, and -γ isomers and that phosphorylation causes a reduction in DNA binding. We also presented evidence that DNA binding by Cut proteins *in vivo* is modulated by phosphorylation and dephosphorylation. Increased DNA binding was observed when nuclear extracts were treated with alkaline phosphatase or when cells were incubated in the presence of calphostin C, a specific inhibitor of PKC (Figs. 4 and 5). Moreover, treatment of cells with the phorbol ester PMA, a specific activator of PKC, led to a rapid reduction in Cut DNA binding and Cut-mediated transcriptional repression (Figs. 6–8). Altogether, these results suggest that Cut repeat DNA binding can be modulated by PKC.

Previous studies on PKC have led to the identification of 11 different isomers, which share a similar catalytic domain but differ in their regulatory domains (28, 31, 41, 42). The presence or absence of certain regulatory domains determines the response to various inducers, including calcium, diacylglycerol, and phorbol esters. Previous studies on the level of PKC activity in a variety of physiological conditions have often led to

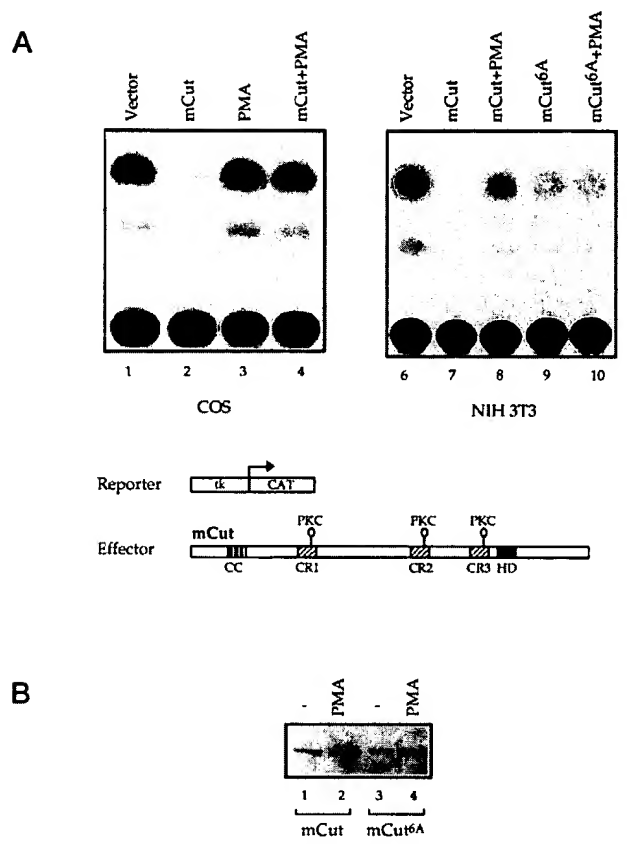


FIG. 8. **Treatment of cells with PMA, an activator of PKC, prevents transcriptional repression by mCut but not by the mutated protein mCut^{6A}.** *A*, COS cells were transfected with 6 μg of reporter plasmid DNA, 9 μg of a vector expressing the β1 subunit of PKC, and 6 μg of either an empty vector or a Cut effector plasmid (mCut). NIH 3T3 cells were transfected with 5 μg of reporter DNA plasmid, a vector expressing the β1 subunit of PKC, and 6 μg of either an empty vector or a Cut effector plasmid encoding the wild type or mutated Cut protein (mCut^{6A}). Forty eight hours later, PMA (100 ng/ml) was added to the medium, and following a 4-h incubation cells were harvested, and cytoplasmic extracts were prepared and processed to measure CAT activity. *B*, NIH 3T3 cells were transfected with 6 μg of either wild type or mutated Cut effector and a vector expressing the β1 subunit of PKC. Cells were stimulated with PMA for 4 h, and Cut expression was then analyzed by Western blot using nuclear extracts and polyclonal antibodies against hCut (2, 9).

contradictory results. However, more recent investigations taking advantage of the isoform-specific reagents now available have clearly established that expression of PKC isozymes is often tissue as well as differentiation stage specific (43). In addition, it has become clear that activation of PKC is accompanied by translocation from the cytosol to various membrane compartments and that the multiple isoforms again differ in their subcellular localization following activation (42–45). In several instances, it has been possible to confirm the involvement of specific PKC isoforms. For example, PKC-α and -δ were shown to promote differentiation of myeloid cells (28). In other cells, differentiation was shown to correlate with an increase in some isoforms, whereas other isoforms were reduced (43). Finally, overexpression of PKC-β, -γ, and -ϵ in rodent fibroblasts was shown to confer one or several of the characteristics associated with cellular transformation (29, 46–49).

Although the identity of the PKC isomer involved in the modulation of Cut activity and the physiological conditions in which this occurs remain to be investigated, some clues can be derived from our current knowledge. Studies on the substrate specificity of PKC isozymes did not reveal major differences in the efficiencies with which various substrates were phosphorylated by the different isozymes (50). On the other hand, there is accumulating evidence to show that individual isoforms can be translocated to specific subcellular compartments on stimulation (42). It appears, therefore, that substrate targeting is likely to be dictated by the subcellular localization of each isozyme following stimulation. Since Cut proteins appear to be localized exclusively to the nucleus (2, 6),[4] only PKC isomers that are translocated to the nuclear membrane would have access to Cut proteins. We cannot exclude that phosphorylation of Cut proteins can take place during the brief period between their translation and their transport to the nucleus. However, this is unlikely, since treatment of cells with PMA leads to a rapid down-regulation of Cut DNA binding, suggesting that at least some of the Cut proteins exist in an unphosphorylated state and become phosphorylated following stimulation of PKC. Several isoforms have been reported to localize to the nuclear membrane, notably α, βI, δ, ϵ, η, and ζ, although discrepancies were noted between different studies (42, 51–55). The decrease in Cut DNA binding following treatment of cells with PMA excludes the involvement of the atypical protein kinase C isozymes λ and ζ, since they do not to respond to phorbol esters (56). Western blot analysis of NIH 3T3 cells has previously shown that only one isoform, PKC-α, was expressed at a detectable level (28, 42). The inhibition of Cut-mediated repression in NIH 3T3 cells transfected with a vector expressing PKC-βI would suggest that PKC-α and -βI are capable of down-modulating Cut activity (Fig. 8). Which one of these isozymes is responsible for this effect and whether other isozymes can modulate Cut in different cellular systems remain to be investigated.

Much progress has been made recently in deciphering the function of Cut proteins at the molecular level. The evolutionarily conserved regions called Cut repeats were found to function as DNA binding domains, and mammalian Cut proteins were shown to act as sequence-specific transcriptional repressors. The biological function of Cut proteins in mammals, however, remains to be defined. Based on the phenotypes of various *cut* mutants and on the effects of overexpressing *cut* in *Drosophila*, it was proposed that Cut is involved in determining cell type specificity in several tissues. Thus, Cut could play a role in several differentiation pathways. The involvement of specific PKC isoforms in some differentiation pathways raises the possibility that one mechanism by which these isoforms contribute to the process of differentiation is through the modulation of Cut activity. In particular, for some pluripotent precursor cells, the treatment of cells with phorbol esters induces differentiation toward one cell type as opposed to another. It is tempting to speculate that the selective inhibition of Cut DNA binding activity following PKC activation is instrumental in determining cell fate. In this respect, it has been shown that the induction of the gp91-*phox* gene during myeloid differentiation correlates with the disappearance of CCAAT displacement protein binding activity. It will be important to verify whether down-modulation of CCAAT displacement protein in this system occurs at the posttranslational level.

We have previously shown that the human Cut protein binds to the promoter of the c-*myc* gene and can repress c-*myc* expression in cotransfection assays. On the other hand, several studies have revealed that PKC-β cooperates with the viral Harvey ras (v-Ha-*ras*) oncogene in cellular transformation and that PKC-β, -γ and -ϵ behave as oncogenes in rodent fibroblasts. Since the c-*myc* oncogene was also found originally to

[4] N. Martin and A. Nepveu, manuscript in preparation.

cooperate with *ras* oncogenes, it is tempting to speculate that one of the roles of PKC isozymes in cellular transformation is to down-modulate Cut DNA binding, thereby preventing repression of c-Myc expression. Future studies should verify whether Cut activity is down-modulated in PKC-transformed fibroblasts. Another issue concerning c-Myc regulation is whether the induction in c-Myc expression following growth factor stimulation of quiescent cells involves the modulation of Cut activity by PKC. We have studied Cut DNA binding activity during the G_0 to G_1 transition in rodent fibroblasts and found that Cut DNA binding activity is absent in quiescent cells.[5] Therefore, we conclude that Cut is not involved in the repression of c-Myc expression in the G_0 phase. It remains to be investigated whether Cut contributes to the repression of c-Myc during cellular differentiation and whether some PKC isoforms are involved in this process.

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